ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 321, No. 2, August 20, pp. 311–318, 1995

ATTACHMENT A

Establishment of a Recombinant Hepatic Cell Line Stably Expressing Alcohol Dehydrogenase

Dahn L. Clemens,[1] Christine M. Halgard, Rodney R. Miles, Michael F. Sorrell, and Dean J. Tuma
Liver Study Unit, Department of Veterans Affairs Medical Center and Department of Internal Medicine, University of Nebraska Medical Center, Omaha, Nebraska 68105

Received January 23, 1995, and in revised form June 1, 1995

Hepatocytes cultured for extended periods of time lose the ability to express alcohol dehydrogenase and thus, the ability to efficiently oxidize ethanol. Therefore, it has been difficult to investigate the effects of chronic ethanol oxidation by hepatocytes *in vitro*. To circumvent this problem, we have inserted the coding region of an exogenous alcohol dehydrogenase gene into an hepatic cell line. Using the human hepatocellular carcinoma cell line, Hep G2, we have constructed an hepatic cell line that stably expresses alcohol dehydrogenase. These recombinant cells, termed HAD 73.1 cells, express approximately 40% of the alcohol dehydrogenase activity of freshly isolated rat hepatocytes. When the ethanol metabolizing ability of these cells was directly measured, the results indicated that not only were these cells able to metabolize ethanol at approximately 70% of the rate of freshly isolated rat hepatocytes but acetaldehyde concentrations of up to 50 μM were detected in the medium. Furthermore, the level of acetaldehyde produced during ethanol oxidation was augmented by cyanamide, an inhibitor of acetaldehyde oxidation, while the ability of these cells to metabolize ethanol was inhibited by pyrazole, an inhibitor of alcohol dehydrogenase. These results suggest that this *in vitro* system will be a valuable tool enabling detailed biochemical studies exploring the effects of chronic ethanol oxidation on the liver and the mechanisms of alcohol-induced hepatic cell injury.

Key Words: **alcohol dehydrogenase; eukaryotic expression; Hep G2; acetaldehyde.**

One of the more serious consequences associated with chronic alcohol abuse is alcoholic liver disease. Alcoholic liver disease is characterized by fatty liver, hepatocyte necrosis, fibrosis, and ultimately cirrhosis. Although the pathology associated with alcoholic liver disease has been well documented, the underlying mechanism(s) of liver injury remains unclear; however, it appears that the detrimental effects of ethanol on the liver are mainly due to ethanol metabolism. One of the major impediments in exploring the mechanisms of alcohol-induced liver cell injury has been the lack of an hepatic tissue culture system to investigate the effects of chronic ethanol oxidation.

The terminally differentiated hepatocytes of the liver oxidize the vast majority of ingested ethanol. This oxidation is mainly the result of the activity of the liver-specific NAD^+-dependent enzyme, alcohol dehydrogenase (ADH)[2] (1, 2). The oxidation of ethanol by alcohol dehydrogenase results in the production of the highly reactive intermediate, acetaldehyde, which in turn is metabolized by aldehyde dehydrogenase to acetate. It has been suggested that the production of acetaldehyde may be responsible for some of the hepatic impairments attributed to the oxidation of ethanol (3–6). Unfortunately, the ability of hepatocytes to efficiently metabolize ethanol, as well as many other liver-specific functions, are rapidly lost in culture (7). Thus, it has been difficult to study the direct effects of alcohol oxidation on hepatic cells *in vitro*. The development of an hepatic cell line capable of stably oxidizing ethanol would enable detailed investigations into the biochemical mechanisms of hepatocyte impairment associated with chronic ethanol oxidation minimizing the confounding variables associated with experimental animal approaches.

We have developed an hepatic cell line that stably and constitutively expresses alcohol dehydrogenase.

[1] To whom correspondence should be addressed. Fax: (402) 449-0604.

[2] Abbreviations used: ADH, alcohol dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PBS, phosphate-buffered saline.

0003-9861/95 $12.00

The recombinant cells, designated HAD cells, were constructed by stably transfecting the hepatocellular carcinoma cell line, Hep G2, with an eukaryotic expression plasmid designed to express alcohol dehydrogenase. The human hepatoblastoma cell line, Hep G2, was chosen as the parental cell line for these experiments because of its phenotypic similarities to hepatocytes (8). Although Hep G2 cells have been shown to synthesize and secrete a number of hepatocyte-specific factors (8, 9), they exhibit very little alcohol dehydrogenase activity (10). The characterization of the recombinant HAD cells in regard to their ability to metabolize ethanol and generate acetaldehyde is the subject of the present study.

MATERIALS AND METHODS

Cells and culture conditions. Hep G2 cells (9) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing high glucose supplemented with 2 mM L-glutamine, 10% fetal bovine serum, and 50 μg/ml gentamicin (complete DMEM). Sodium pyruvate was normally present at 1 mM, although for some experiments sodium pyruvate was absent or present at 10 mM. Recombinant HAD cells were cultured in complete DMEM containing 200 μg/ml hygromycin B. All cells were maintained at 37°C in a humidified environment containing 5% CO_2. During ethanol and acetaldehyde metabolism studies, the growth media were supplemented with the indicated concentrations of ethanol or acetaldehyde, 25 mM Hepes, pH 7.3, and the cells were cultured in tightly sealed flasks to minimize evaporation of ethanol and acetaldehyde. Rat hepatocytes were isolated by a modification of the collagenase perfusion method of Seglen (11) as previously described by Casey *et al.* (12).

Establishment of recombinant HAD cell lines. Hep G2 cells were stably transfected with pIC-14, an eukaryotic expression plasmid containing a cDNA copy of the murine alcohol dehydrogenase gene, *Adh-1* (13, 14). The expression plasmid pIC-14 was constructed in a two step cloning procedure. First, the alcohol dehydrogenase cDNA was excised from pHel-66 (a kind gift from Dr. Howard Edenberg) by digestion with *Hin*dIII and *Sma*I. The 1.7-kb alcohol dehydrogenase containing fragment was isolated from the gel, purified, and inserted into the corresponding sites of pSP72 (Promega Corp.). The resulting plasmid was designated pIC-6. This step was necessary to facilitate the insertion of the alcohol dehydrogenase coding region in the proper transcriptional orientation with respect to the cytomegalovirus immediate early promoter in the eukaryotic expression vector pCEP4 (Invitrogen Corp.). Finally, pIC-14 was constructed by excising the alcohol dehydrogenase coding region from pIC-6 with *Hin*dIII and *Kpn*I, isolating the 1.7-kb alcohol dehydrogenase fragment and inserting it into the corresponding sites of pCEP4.

The recombinant plasmid, pIC-14, was transfected into Hep G2 cells by the calcium phosphate method essentially as described by Graham and Van Der Eb (15). Recombinant cells were selected in the presence of 200 μg/ml hygromycin B: clones were isolated and clonal cell lines established as previously described (16). Twenty-six clonal cell lines were isolated, expanded into cell lines, and screened for alcohol dehydrogenase activity. The cell line designated HAD 73.1 showed the greatest alcohol dehydrogenase activity and has been used in all of the studies described below.

Ribonuclease protection assays. Total cellular RNA was isolated from cultured cells and homogenized liver tissue by the method of Chomczynski and Sacchi (17). Briefly, cells were washed twice in PBS and resuspended in 4 M guanidinium thiocyanate. The lysate was extracted with phenol/chloroform/isoamyl alcohol (24:24:1) and then precipitated with isopropanol. RNA pellets isolated from tissue were washed once in 4 M LiCl to remove glycogen before being resuspended in 4 M guanidinium thiocyanate and reprecipitated.

Negative sense [^{32}P]UTP-labeled riboprobes were produced from linearized plasmid constructs using Ambion's MAXIscript *in vitro* transcription kit according to the manufacturer's instructions.

Ambion's RPA II kit was used to perform ribonuclease protection assays on total cellular RNA isolated from HAD 73.1 and Hep G2 cells, as well as murine and human hepatocytes. Briefly, 5 μg of HAD 73.1 and Hep G2 RNA and 2 μg of hepatocyte RNA were hybridized to 8×10^5 cpm of probe at 42°C overnight in 80% deionized formamide, 100 mM sodium citrate (pH 6.4), 300 mM sodium acetate (pH 6.4), and 1 mM EDTA. Following hybridization, samples were digested with 0.05 units of RNase A and 2 units of RNase T for 30 min at 37°C. Protected fragments were separated on 5% polyacrylamide gels containing 8 M urea and visualized by exposing the gels to X-ray film for 2 days. Probes protected 513, 316, 301, and 250 bases for alcohol dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), cytochrome P450IIE1, and β-actin target mRNA's, respectively.

Alcohol dehydrogenase activity. Alcohol dehydrogenase activity was determined by a modification of the procedure described by Crow *et al.* (18). Briefly, cells were seeded on 75-cm^2 flasks at 8×10^6 cells/flask and incubated at 37°C for 48 h. Following the incubation, the growth media was removed and the cell sheet was washed with PBS. The cells were lysed on the flask by addition of 1 ml of 1% Triton X-100. Lysates from freshly isolated hepatocytes were prepared in a similar fashion in culture tubes. The lysates were then sonicated five times for 5 s prior to assaying. The alcohol dehydrogenase assay was performed by adding 250 μl of lysate to 10 mM ethanol, 3 mM NAD in 1-ml volume buffered with 0.5 M Tris–HCL, pH 7.4. The reaction components were prewarmed to 37°C prior to the addition of the ethanol and the change in optical density at 340 nm was monitored using a Beckman DU-70 spectrophotometer. We have expressed alcohol dehydrogenase activity as nmol of NADH generated/h/mg total cellular protein. Following each assay the total cellular protein present was determined using the BCA Protein Assay Reagent (Pierce Chemical Company).

Aldehyde dehydrogenase activity. Aldehyde dehydrogenase activity was determined by a modification of the procedure described by Cao *et al.* (19). Briefly, cells were seeded and cultured as described above, the growth media was removed and the cell sheet was washed with PBS. The cells were collected in 1 ml of reaction buffer containing 100 mM $NaPO_4$, pH 7.4, 3 mM NAD, and 10 mM pyrazole. The samples were then sonicated five times for 5 s and Triton X-100 was added to a final concentration of 1% prior to assaying. Lysates from freshly isolated hepatocytes were prepared in a similar fashion in culture tubes. The enzyme assay was performed at 25°C and was initiated by the addition of propionaldehyde to a final concentration of either 25 μM (low K_m enzyme) or 1 mM (total enzyme activity). Aldehyde dehydrogenase activity was determined by the change in optical density at 340 nm. We have expressed aldehyde dehydrogenase activity as nmol of NADH generated/h/mg total cellular protein. Following each assay the total cellular protein present was determined as described above.

Ethanol metabolism. Ethanol metabolism was determined by the elimination of ethanol from the growth media. Briefly, 25-cm^2 flasks were seeded at 5×10^6 cells/flask and incubated overnight at 37°C. The following morning the growth media was removed and replaced with complete DMEM containing ethanol and Hepes. Samples were taken from the tightly sealed flasks at various time points and ethanol and acetaldehyde concentrations determined by headspace gas-chromatography as previously described (20). In some experiments alcohol dehydrogenase and aldehyde dehydrogenases were inhibited by the addition of 2 mM pyrazole (21, 22) or 0.1 mM cyanamide (23, 24), respectively, to the growth medium.

TABLE I

Alcohol Dehydrogenase Activity and Rates of Ethanol Metabolism in Recombinant HAD 73.1 Cells, Hep G2 Cells, and Rat Hepatocytes

Sample	Alcohol dehydrogenase activity[a]	Percentage of hepatocytes	Ethanol metabolized[b]	Percentage of HAD 73.1	Percentage of hepatocytes
Hep G2	3.5 ± 0.87	0.5	18.4 ± 3.6	7.7	5.3
HAD 73.1	234 ± 18.1	38.5	240.1 ± 4.2	100	69.1
Fresh rat hepatocytes	607 ± 26.9	100	347.6 ± 22.5	125	100

[a] Alcohol dehydrogenase activity is expressed as nmol of NAD+ oxidized/h/mg protein and shown as the mean ± the standard error of the mean (SEM) of 12 samples.

[b] Ethanol metabolism data are presented as nmol of ethanol cleared/h/mg protein and shown as the mean ± SEM of 12 samples.

RESULTS

Hep G2 cells were transfected with pIC-14. The plasmid pIC-14 contains the murine *Adh-1* coding region inserted downstream of the cytomegalovirus immediate early promoter/enhancer in the eukaryotic expression vector pCEP4. Additionally, pCEP4 contains the bacterial hygromycin B phosphotransferase gene regulated by the herpes simplex virus thymidine kinase promoter, which functions as a dominant selectable marker in eukaryotic cells. Following transfection, the cells were cultured in the presence of hygromycin B. Twenty-one days after transfecting the cells, small macroscopic colonies were visible and independently isolated.

Initially, isolated clonal cell lines were expanded and assayed for alcohol dehydrogenase activity. Of the twenty-six cell lines established, HAD 73.1 showed the greatest alcohol dehydrogenase activity. Therefore, this recombinant cell line was chosen as the focus of further study. Detailed analysis of lysates from this cell line revealed that HAD 73.1 cells had approximately 67 times more alcohol dehydrogenase activity than the parental Hep G2 cell line (Table I). Comparison of the alcohol dehydrogenase activity of HAD 73.1 cells to freshly isolated rat hepatocytes indicated that HAD 73.1 cells exhibited approximately 40% of the alcohol dehydrogenase activity of freshly isolated rat hepatocytes (Table I). In comparison, the parental cell line, Hep G2, had approximately 0.5% of the alcohol dehydrogenase activity of freshly isolated rat hepatocytes.

To determine if the alcohol dehydrogenase activity observed in the HAD 73.1 cells was a result of transcriptional activity of the exogenous murine alcohol dehydrogenase cDNA, ribonuclease protection assays were performed. The results of these assays revealed a predominant band in the HAD 73.1 sample that comigrated with the predominant band in a murine liver sample (Fig. 1). This band was absent in both the Hep G2 and human liver samples (although a much smaller band was detected in the human liver sample, data not shown). Furthermore, when assaying HAD 73.1 and Hep G2 samples with a probe specific for human cytochrome P450IIE1, we were unable to detect transcription of the gene in either sample. Hybridization of the HAD 73.1 and Hep G2 samples with either a GAPDH or β-actin probe revealed that similar amounts of HAD 73.1 and Hep G2 RNA were analyzed (data not shown). These results indicate that the alcohol dehydrogenase activity observed in the recombinant HAD 73.1 cells was likely due to the expression of the exogenous murine alcohol dehydrogenase cDNA.

We next investigated the ability of HAD 73.1 cells to metabolize ethanol directly. Both HAD 73.1 cells and control Hep G2 cells were cultured in the presence of 10 mM ethanol and samples removed at various times over the course of 82 h. The results shown in Fig. 2 indicate that there was a dramatic difference in the ability of the two cell lines ability to metabolize ethanol. HAD 73.1 cells completely metabolized this concentration of ethanol within 82 h, whereas, very little ethanol had been metabolized by the Hep G2 cells during this same time period.

To insure that the metabolic activity observed in HAD 73.1 cells was in fact due to alcohol dehydroge-

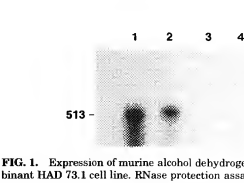

FIG. 1. Expression of murine alcohol dehydrogenase in the recombinant HAD 73.1 cell line. RNase protection assays were performed using a 513-base murine alcohol dehydrogenase antisense [^{32}P]UTP-labeled riboprobe and 5 μg of total RNA isolated from HAD 73.1 cells and Hep G2 cells or 2 μg of total RNA isolated from murine and human hepatocytes. Lane 1, murine hepatocytes; lane 2, HAD 73.1 cells; lane 3, Hep G2 cells; lane 4, human hepatocytes.

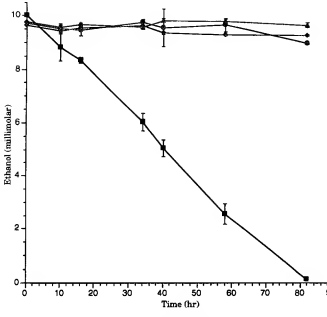

FIG. 2. Ethanol metabolism by recombinant HAD cells. The concentration of ethanol in cultures of HAD 73.1 cells (♦) and Hep G2 cells (●) cultured in the presence of 2 mM pyrazole and HAD 73.1 cells (■) and Hep G2 cells (▲) cultured in the absence of 2 mM pyrazole was monitored. Samples were removed at the indicated time points and the ethanol concentrations determined by headspace gas chromatography. The data are expressed as means of four experiments ± the SEM.

nase, we monitored the ability of both the HAD 73.1 and Hep G2 cells to metabolize ethanol in the presence of the potent alcohol dehydrogenase inhibitor, pyrazole. The results also depicted in Fig. 2 show that inclusion of 2 mM pyrazole in the culture media dramatically inhibited the ability of HAD 73.1 cells to efficiently metabolize ethanol, effectively reducing their ethanol metabolizing capacity to that of the parental Hep G2 cell line (Fig. 2). Because complete DMEM normally contained 1 mM pyruvate, and pyruvate has been shown to augment the ability of hepatocytes to metabolize ethanol (18), we repeated these experiments in the presence of 10 mM pyruvate or in its complete absence. We observed no difference in the ability of these cells to metabolize ethanol under these conditions (data not shown). Together these results indicate that HAD 73.1 cells had a substantially greater capacity to metabolize ethanol than Hep G2 cells and that this capability was mediated by alcohol dehydrogenase. Comparison of HAD 73.1 cells to freshly isolated rat hepatocytes indicated that HAD cells metabolized ethanol at approximately 70% of the rate of freshly isolated rat hepatocytes (Table I). This is in contrast to the alcohol dehydrogenase activity observed in HAD cell lysates (Table I), which was approximately 40% of that observed in rat hepatocyte lysates.

The highly reactive compound, acetaldehyde, is the first metabolite produced by the alcohol dehydrogenase-mediated oxidation of ethanol. Because acetaldehyde has been implicated as a mediator of ethanol-induced hepatic cell injury (5, 6), it was of interest to determine the levels of acetaldehyde produced by HAD 73.1 cells. The data shown in Fig. 3 indicated that acetaldehyde concentrations during ethanol oxidation rapidly rose, reaching 50 μM by 28 h and then gradually decreased to basal levels by 90 h.

In an attempt to determine the cause of the transient nature of the relatively high acetaldehyde concentrations, a similar experiment using 20 mM ethanol was performed to ensure that the ethanol concentrations remained substantially greater than the K_m of alcohol dehydrogenase throughout the sampling period. Although culturing HAD 73.1 cells in 20 mM ethanol appeared to augment the acetaldehyde levels, a similar pattern of acetaldehyde elimination was observed (Fig. 4), suggesting that the decrease in acetaldehyde concentrations upon prolonged incubation was not solely due to the decrease in ethanol available for metabolism.

To investigate the cause of the gradual decrease in acetaldehyde concentrations with time despite a constant rate of ethanol metabolism, we determined the level of aldehyde dehydrogenase activity in HAD 73.1 cells either prior to or following ethanol exposure. The results of these experiments revealed that HAD 73.1 cells exhibited approximately 40.8 ± 7.8 nmol/h/mg total cellular protein of low K_m aldehyde dehydrogenase activity and 75.6 ± 16 nmol/h/mg protein of total aldehyde dehydrogenase activity. Following ethanol exposure, the values were determined to be 38.5 ± 5.6 nmol/h/mg protein and 94.2 ± 15.4 nmol/h/mg for the low K_m and total aldehyde dehydrogenase activity, respectively. This activity compares to 177.8 ± 38.4 nmol/h/mg protein and 583.1 ± 92.1 nmol/h/mg protein for the low K_m and total aldehyde dehydrogenase activity, respectively, which was observed in freshly isolated rat hepatocytes. These data suggest that there is no difference in aldehyde dehydrogenase activity following ethanol exposure, and cannot by themselves account for the observed decrease in acetaldehyde.

We also investigated the ability of naive HAD 73.1 cells and HAD 73.1 cells exposed to ethanol for 24 h to clear exogenous acetaldehyde from the culture media. There was no difference in the acetaldehyde clearance between these two groups (Fig. 5), again suggesting that the apparent increased disappearance of acetaldehyde was not due to an increase in the activity of aldehyde dehydrogenase. Furthermore, to determine if the alcohol dehydrogenase activity of the HAD cells had any effect on the clearance of exogenous acetaldehyde via its reduction back to ethanol, we investigated the ability of HAD 73.1 cells to metabolize acetaldehyde in the presence and absence of pyrazole. There was no

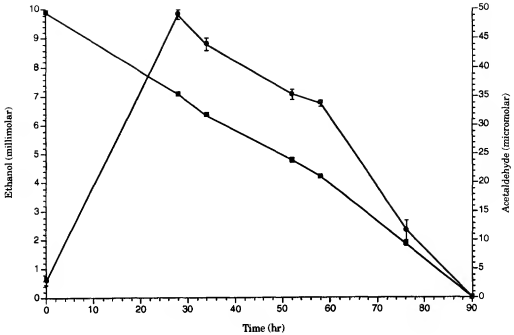

FIG. 3. Acetaldehyde production in HAD cell cultures. HAD 73.1 cells were cultured in the presence of 10 mM ethanol and samples removed at the indicated time points. Both the ethanol (■) and acetaldehyde (●) concentrations were determined by headspace gas chromatography. The data are expressed as means of 12 experiments ± the SEM.

difference in the ability of these two groups to metabolize acetaldehyde (data not shown).

In an attempt to determine if the levels of endogenous acetaldehyde produced by HAD 73.1 cells could be increased, the ethanol metabolism experiment was repeated in the presence of cyanamide, an inhibitor of aldehyde dehydrogenase. The results of this experiment shown in Fig. 6 revealed that ethanol metabolism by HAD 73.1 cells cultured in the presence of cyanamide resulted in acetaldehyde levels three to four times greater than in HAD 73.1 cells cultured in its absence. The augmented levels of acetaldehyde produced by the cells cultured in the presence of cyanamide were sustained for 30 h and then gradually decreased. Hep G2 cells were included as controls in these experiments, they metabolized little ethanol and produced undetectable levels of acetaldehyde (data not shown).

DISCUSSION

Hepatocytes cultured *in vitro* for extended periods lose the ability to express many of the liver-specific functions that they perform *in vivo*. Among the attributes lost in cultured hepatocytes are the expression of alcohol dehydrogenase and the ability to efficiently metabolize ethanol. Thus, it has been difficult to study the effects of chronic ethanol oxidation in cultured cells. Recently, Mapoles *et al.* (25) reported the establishment of a chinese hamster ovary cell line, which stably oxidizes ethanol and produces acetaldehyde. In an attempt to establish a suitable *in vitro* model that more closely resembles hepatic ethanol oxidation, we have inserted the coding region of the murine liver *Adh-1* alcohol dehydrogenase gene into the human hepatoma cell line Hep G2. Although Hep G2 cells are phenotypically similar to hepatocytes in that they synthesize and secrete many plasma proteins normally produced by hepatocytes (8, 9), they express very little alcohol dehydrogenase (10). Conversely, the recombinant HAD 73.1 cells, developed in this study, express approximately 40% of the alcohol dehydrogenase of freshly isolated rat hepatocytes and metabolize ethanol approximately 70% as efficiently as freshly isolated rat hepatocytes.

Comparing the alcohol dehydrogenase data with the ethanol metabolism data, it appears HAD 73.1 cells express alcohol dehydrogenase (234 nmol/h/mg protein) as efficiently as they metabolize ethanol (240 nmol/h/mg protein). In contrast, the freshly isolated rat hepatocytes exhibit approximately 610 nmol/h/mg protein of alcohol dehydrogenase activity but metabolize approximately 350 nmol/h/mg protein of ethanol. Thus, our data suggest that the metabolism of ethanol in HAD 73.1 cells is limited by alcohol dehydrogenase activity while the metabolism of ethanol in freshly isolated hepatocytes is not. Consistent with this conclusion is the fact that pyruvate was unable to augment the rate of ethanol metabolism in HAD 73.1 cells, but

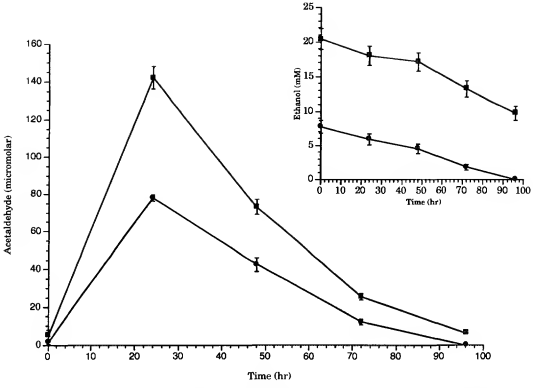

FIG. 4. Comparison of acetaldehyde produced in HAD 73.1 cell cultures as a function of ethanol concentration. HAD 73.1 cells were cultured in either 7.5 mM (●) or 20 mM (■) ethanol. Samples were taken at the indicated time points and the acetaldehyde concentrations were determined by headspace gas chromatography. The insert shows the clearance of ethanol under these two cultures conditions during sampling. The data are expressed as means of four experiments ± the SEM.

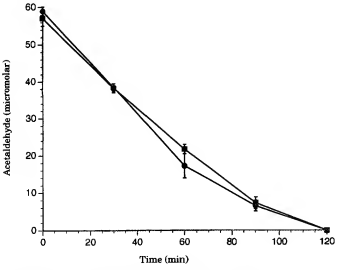

FIG. 5. Clearance of exogenous acetaldehyde by HAD cells. HAD 73.1 cells were exposed to 60 μM acetaldehyde either before (■) or after (●) 24 h ethanol exposure as described under Materials and Methods. Samples were taken at the indicted time points and acetaldehyde concentrations determined by headspace gas chromatography. The data are expressed as means of four experiments ± the SEM.

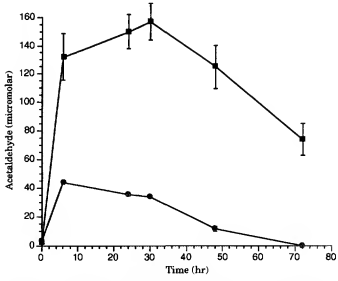

FIG. 6. Acetaldehyde production in HAD 73.1 cell cultures in the presence of cyanamide. HAD 73.1 cells were cultured in 10 mM ethanol in the presence (■) or the absence (●) of 0.1 mM cyanamide. Samples were taken at the indicated time points and the acetaldehyde concentrations were determined by headspace gas chromatography. The data are expressed as means of four experiments ± the SEM.

has been shown to do so in isolated hepatocytes. Therefore, it appears that some other factor(s), perhaps the availability of the cofactor NAD^+ limits ethanol metabolism in isolated hepatocytes. Furthermore, the similarities between the expression of alcohol dehydrogenase and the metabolism of ethanol by HAD 73.1 cells suggests that other ethanol metabolizing pathways (e.g., cytochrome P450 and catalase) are not major contributors to the activity observed in HAD cells.

We have chosen to express the ethanol metabolism data as nmol of ethanol oxidized/h/mg of total protein. Expressing the data in these terms indicates HAD 73.1 cells metabolized ethanol approximately 70% as efficiently as control hepatocytes. It should be pointed out that HAD cells are considerably smaller than hepatocytes, and therefore, when compared on a per cell basis, HAD cells metabolize much less ethanol than hepatocytes (approximately 15–20%).

Acetaldehyde is the first product of alcohol dehydrogenase-mediated oxidation of ethanol. Acetaldehyde is a very reactive compound and has been shown to bind various hepatic proteins (26, 27) and affect a number of cellular functions, including DNA repair (28), microtubule assembly (29), fatty acid oxidation (30), and mitochondrial respiration (31). Thus, it has been proposed that acetaldehyde is a mediator of alcohol-induced hepatic cell toxicity.

We have shown in this study that acetaldehyde is produced by HAD 73.1 cells, rapidly reaching a peak of approximately 50 μM and then gradually decreasing to basal levels. These micromolar levels of acetaldehyde are similar to those reported in the liver of animals (32) and humans following ethanol exposure (33). Furthermore, our results indicate that the levels of acetaldehyde produced by HAD 73.1 cells can be manipulated by using the aldehyde dehydrogenase inhibitor, cyanamide. Thus, this system may to be an excellent model for the study of acetaldehyde-mediated hepatic cell toxicity.

One finding associated with the oxidation of ethanol by HAD 73.1 cells was that the levels of acetaldehyde detected in cells exposed to ethanol gradually decrease over time. This decrease did not appear to result from reduced oxidation of ethanol by HAD 73.1 cells or a decrease in the rate of acetaldehyde production. We also determined that ethanol exposure did not increase aldehyde dehydrogenase activity in these cells or the ability of these cells to clear acetaldehyde. Furthermore, we determined that the disappearance of the majority of the acetaldehyde was indeed the result of oxidation of acetaldehyde by aldehyde dehydrogenase because inhibiting aldehyde dehydrogenase with cyanamide altered the rapid decrease in acetaldehyde levels.

As mentioned above, the activity of aldehyde dehydrogenase observed in the HAD 73.1 cells cannot entirely account for the decrease in acetaldehyde. It has been suggested that an inability to account for all the acetaldehyde produced by the oxidation of ethanol may be a result of its covalent binding to proteins, rendering it unavailable for standard detection methods (34). We have detected the presence of acetaldehyde-protein adducts in HAD cells chronically exposed to ethanol (D. L. Clemens and D. J. Tuma, unpublished results). Thus the formation of acetaldehyde–protein adducts may also account for the apparent disappearance of some acetaldehyde produced during the oxidation of ethanol by HAD 73.1 cells. Experiments designed to further investigate the apparent decrease in the levels of acetaldehyde are currently under way.

In summary, we have established a cell line of hepatic origin that stably and constitutively expresses alcohol dehydrogenase. These cells designated HAD 73.1 cells efficiently metabolize ethanol and produce acetaldehyde. Construction and use of these cells will allow a differentiation between the hepatotoxic effects of ethanol itself and its oxidation products. Thus, it is hoped that these HAD cells will be useful in exploring the mechanism(s) that underlie many of the hepatic impairments associated with the oxidation of ethanol and the formation of acetaldehyde–protein adducts.

ACKNOWLEDGMENTS

This work was supported by the Omaha Veterans Administration Alcohol Research Center and a grant from the University of Nebraska Medical Center. We also thank Dr. Howard Edenberg for the kind gift of pHel-66.

REFERENCES

1. Crabb, D. W., Bosron, W. F., and Li, T. K. (1983) *Arch. Biochem. Biophys.* **224,** 299–309.
2. Ehrig, T., Borson, W. F., and Li, T. K. (1990) *Alcohol Alcohol.* **25,** 105–116.
3. Lieber, C. S. (1988) *Biochem. Soc. Trans.* **16,** 241–247.
4. Jennett, R. B., Tuma, D. J., and Sorrell, M. F. (1990) *in* Progress in Liver Disease (Popper, H., and Schaffner, F., Eds.), Vol. IX, pp. 325–333, Saunders, Philadelphia.
5. Sorrell, M. F., and Tuma, D. J. (1985) *Alcohol. Clin. Exp. Res.* **9,** 306–309.
6. Volentine, G. D., Ogden, K. A., Kortje, D. K., Tuma, D. J., and Sorrell, M. F. (1987) *Hepatology* **7,** 490–495.
7. Lea, M. A. (1993) *Int. J. Biochem.* **25,** 457–469.
8. Knowles, B. B., Searls, D. B., and Aden, D. P. (1984) *in* Advances in Hepatitis Research (Chisari, F. V., Ed.), pp. 196–202, Masson, New York.
9. Knowles, B. B., Howe, C. C., and Aden, D. P. (1980) *Science* **209,** 497–499.
10. Wolfla, C. E., Ross, R. A., and Crabb, D. W. (1988) *Arch. Biochem. Biophy.* **263,** 69–76.
11. Seglen, P. O. (1988) *Methods Cell Biol.* **13,** 29–83.
12. Casey, C. A., Kragskow, S. L., Sorrell, M. F., and Tuma, D. J. (1987) *J. Biol. Chem.* **262,** 2704–2710.
13. Edenberg, H. J., Zhang, K., Fong, K., Bosron, W. F., and Li, T. K. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 2262–2266.

14. Ceci, J. D., Zheng, Y. W., and Felder, M. R. (1987) *Gene* **59,** 171–182.
15. Graham, F. L., and Van Der Eb, A. J. (1973) *Virology* **52,** 456–467.
16. Clemens, D. L., and Carlson, J. O. (1989) *J. Virol.* **63,** 2737–2745.
17. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
18. Crow, K. E., Cornell, N. W., and Veech, R. L. (1977) *Alcohol. Clin. Exp. Res.* **1,** 42–50.
19. Cao, Q-N., Tu, G-C., and Weiner, H. (1989) *Biochem. Pharm.* **38,** 77–83.
20. Tuma, D. J., Zetterman, R. K., and Sorrell, M. F. (1980) *Biochem. Pharmacol.* **29,** 35–38.
21. Grunnet, N., Quistorff, B., and Thieden, H. I. D. (1973) *Eur. J. Biochem.* **40,** 275–282.
22. Theorell, H., and Yonetani, T. (1963) *Biochem. Z.* **338,** 537–553.
23. Marchner, H., and Tottman, O. (1978) *Acta. Pharmacol. Toxicol.* **43,** 219–232.
24. Cederbaum, A. I., and Dicker, E. (1981) *Biochem. Pharmacol.* **30,** 3079–3088.
25. Mapoles, J. E., Iwahashi, M., Lucus, D., Zimmerman, B. T., and Simon, F. R. (1994) *Alcohol. Clin. Exp. Res.* **18,** 632–639.
26. Medina, V., Donahue, T. M., Sorrell, M. F., and Tuma, D. J. (1985) *J. Lab. Clin. Med.* **105,** 5–10.
27. Worrall, S., De Jersey, J., Shanley, B. C., and Wilce, P. A. (1991) *Clin. Invest.* **87,** 1367–1374.
28. Espina, N., Lima, V., and Lieber, C. S. (1988) *Carcinogenesis* **9,** 761–766.
29. Jennett, R. B., Tuma, D. J., and Sorrell, M. F. (1980) *Pharmacology* **21,** 363–368.
30. Matsuzaki, S., and Lieber, C. S. (1977) *Biochem. Biophys. Res. Commun.* **75,** 1059–1065.
31. Cederbaum, A., Lieber, C. S., and Rubin, E. (1974) *Arch. Biochem. Biophys.* **161,** 26–39.
32. Pikkarainen, P. H., Salaspuro, M. P., and Lieber, C. S. (1979) *Alcohol. Clin. Exp. Res.* **3,** 259–261.
33. Lindros, K. O., Stowell, A., Pikkarainen, P. H., and Salaspuro, M. (1980) *Pharmacol. Biochem. Behav.* **13,** 119–124.
34. Nuutinen, H., Lindros, K. O., and Salaspuro, M. (1983) *Alcohol. Clin. Exp. Res.* **7,** 163–168.
35. Svanas, W., and Weiner, H. (1985) *Arch. Biochem. Biophys.* **236,** 36–46.